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Utility of arginine kinase for resolution of phylogenetic relationships among brachyuran genera and families

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ABSTRACT

The molecular phylogenetics of decapod crustaceans has been based on sequence data from a limited number of genes. These have included rapidly evolving mitochondrial genes, which are most appropriate for studies of closely related species, and slowly evolving nuclear ribosomal RNA genes, which have been most useful for resolution of deep branches within the Decapoda. Here we examine the utility of the nuclear gene that encodes arginine kinase for phylogenetic reconstruction at intermediate levels (relationships among genera and families) within the decapod infraorder Brachyura (the true crabs). Analyses based on arginine kinase sequences were compared and combined with those for the mitochondrial cytochrome oxidase I gene. All of the genera in our taxon sample were resolved with high support with arginine kinase data alone. However, some of these genera were grouped into clades that are in conflict with recognized brachyuran families. A phylogeny based on cytochrome oxidase I was consistent with the arginine kinase phylogeny, but with weaker support. A recently proposed measure of phylogenetic informativeness indicated that arginine kinase was generally more informative than cytochrome oxidase I for relationships above the level of genus. Combined analysis of data from both genes provided strong support for clades that are in conflict with current assignments of genera to the families Epialtidae, Mithracidae, Pisidae, and Portunidae.

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1. Introduction

The crustacean order Decapoda is an ecologically and economically important group that includes crabs, shrimps, lobsters and crayfish. Phylogenetic relationships within the Decapoda have been the subjects of numerous molecular systematic investigations. Some of the earliest were based on the nuclear large subunit ribosomal RNA gene (18S), and focused on relationships among suborders and families (Kim and Abele, 1990; Perez-Losada et al., 2002; Spears et al., 1992). After Cunningham and co-workers (1992) used a phylogeny based on the mitochondrial large subunit ribosomal RNA gene (16S) to argue that lithodid crabs were part of a paguroid clade, 16S became widely used for crustacean molecular systematics. Unlike 18S, 16S evolves rapidly enough to resolve phylogenetic relationships among closely related species (Schubart et al., 2000). For some studies of decapod systematics, data from multiple ribosomal RNA genes have been combined (e.g. Ahyong and O'Meally, 2004; Tudge and Cunningham, 2002). A few of the protein-coding genes of the mitochondrial genome have also been used in decapod systematics. The gene that encodes cytochrome oxidase subunit I (COXI) is used both in decapod phylogenetics and also as a "DNA barcode" for species identification (Hebert et al., 2003). Cytochrome oxidase II has been used for the molecular systematics of crabs in the family Aeglidae (Perez-Losada et al., 2004).

Relatively few phylogenetic analyses of decapods have used the sequences of protein-coding genes from the nuclear genome. A phylogenetic analysis based on glucose-6-phosphate isomerase (GPI) and elongation factor-1α (EF-1α) along with the mitochondrial protein-coding gene cytochrome oxidase subunit I (COXI) provided evidence of three major clades within the snapping shrimp genus *Alpheus* (Williams et al., 2001). Porter et al. (2005) combined data for three ribosomal genes (16S, 18S and the nuclear 28S gene) with sequences of the nuclear histone H3 gene to examine relationships among decapod infraorders. Regier and Shultz (2001) demonstrated the usefulness of the nuclear protein-coding gene elongation factor-2 (EF-2) to resolve higher-level relationships within the Arthropoda, such as the controversial relationships among the subphyla Crustacea, Hexapoda and Myriapoda.

For accurate phylogenetic inference, gene sequences must be correctly aligned and provide a phylogenetic signal that is not degraded by saturation at variable sites. Of the genes that have been most commonly used for crustacean systematics, ribosomal RNA sequences can be difficult to align without ambiguity and mitochondrial protein-coding genes often prone to mutational saturation. Neigel and Mahon (2007) argued that there has been

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too much reliance on these few genes in crustacean systematics and that additional nuclear protein-coding loci are needed to generate accurate phylogenies. In their review, they noted that of the 14 loci that have been used for crustacean systematics, five are mitochondrial and four are within the nuclear ribosomal repeat unit. Of the remaining five, four encode proteins with highly conserved amino acid sequences that are most useful for resolution of higher-level relationships within the Arthropoda. Only one gene, glucose 6-phosphate isomerase, qualifies as a nuclear gene that encodes a protein that is not highly conserved at the amino acid level (Williams et al., 2001). In contrast, over 30 nuclear protein-coding loci have been used in insect systematics (Caterino et al., 2000; Hardy, 2007). Clearly, there is a need for more protein-coding nuclear genes in crustacean systematics, especially for questions about phylogenetic relationships at levels that fall between the optimal ranges of highly conserved nuclear genes and rapidly evolving mitochondrial genes.

The average rate of sequence divergence can provide a rough indication of how suitable a gene might be for the resolution of deep versus shallow phylogenetic relationships. However, because the actual distribution of rates among sites is critical, the average rate is not a reliable measure of how useful a gene sequence will be for a particular phylogenetic problem. Townsend (2007) recently proposed such a measure, phylogenetic informativeness (PI), based on the number of sites expected to have changed along the short internal branches of a polytomy but not along the long branches that lead from the polytomy to the terminal taxa. PI is calculated from estimated rates of change at each site rather than an average rate for the entire sequence. Thus a sequence with a few rapidly evolving sites among many invariant sites will have a different PI than a sequence with the same overall rate of divergence but a more even distribution across sites. Profiles of relative phylogenetic informativeness (RPI) can be used to show the proportional contributions of different genes to the resolution of polytomies across a range of depths within a phylogeny.

Here we evaluate a region of the nuclear gene that encodes arginine kinase (AK) for phylogenetic analysis of the Brachyura. AK belongs to the phosphagen kinase family of enzymes, which catalyze the reversible transfer of phosphoryl groups between ATP and phosphagen energy stores (Ellington, 2001). This activity functions to buffer ATP levels under conditions of high demand (Meyer et al., 1984; Walliman et al., 1992), regulate levels of inorganic phosphate (Meyer et al., 1986), and transport energy intracellularly (Bessman and Capenter, 1985). AK has been identified in most animal groups, except vertebrates, although AK in echinoderms appears to have evolved independently of AK in other animals (Suzuki et al., 1999). We conducted our phylogenetic analysis with AK sequences in parallel with an analysis based on the mitochondrial gene COXI. This allowed us to compare our results for AK with those for one of the most commonly used genes in crustacean systematics.

2. Materials and methods

2.1. Specimen collection

Live specimens were collected by snorkeling or SCUBA, or purchased from commercial vendors (Table 1). Tissue was removed immediately after animals were sacrificed so that DNA could be extracted from fresh tissue that was not frozen or preserved in alcohol. Most specimens were photographed, preserved as vouchers and deposited in our collection.

2.2. Taxon sampling

Taxa were selected to represent groupings at several levels: species within genera, genera within families, and families within superfamilies (Table 1). We used the brachyuran section of Martin and Davis (2001) for an overall taxonomic framework and McLaughlin and colleagues (2005) for individual assignments of species to families. Based on these classifications, our taxon sample for AK sequences includes five species within the genus *Cancer*, seven species representing four families within the superfamily Majoidea, six species representing two families within the superfamily Xanthoidea, and nine species representing three families within the superfamily Grapsoidea. We also included representatives of the families Portunidae, Ocypodidae and Pinnotheridae. Sequences from two non-brachyuran decapod species were used as outgroups: the anomuran *Oedignathus inermis* and the thalassinidean *Lepidopthalmus louisianensis*.

Our sample of COXI sequences included most, but not all of the species included in our taxon sample for AK (Table 1). Except where sequences where obtained from GenBank, AK and COXI sequences were obtained from the same specimen. For phylogenies that were based all or in part on COXI sequences, we used *O. inermis* as the sole outgroup. We did not use *L. louisianensis* in these cases because its COXI sequence exhibited unusual (for decapods) biases in nucleotide composition; which have been shown to produce errors in phylogenetic inference (Hassanin et al., 2005).

2.3. Primer design

PCR primers for AK were based on alignment of cDNA sequences from several species (GenBank Accession Nos.: AF167313, AF233355, AF233356, AF233357, and AF288785). We used the computer program NAR (Rychlik and Rhoads, 1989) to avoid primer sequences with significant secondary structure or potential for dimer formation. Two pairs of primers were designed to amplify overlapping regions of the second exon of the AK gene (Fig. 1). The first pair were designated AKF2 (forward) and AKR2 (reverse) and the second pair AKF3 (forward) and AKR3 (reverse). AKR3 is a modification of the ENDR1 primer (Kotlyar et al., 2000) designed for amplification of AK cDNA from *Carcinus maenas* and *Callinectes sapidus*. Degenerate primers for amplification of a portion of the COXI gene (primers COIF2 and COIR2, product size 658 bp or COIF2B and COIR2, 622 bp) were based on primers commonly used for insects (Simon et al., 1994), which we modified for brachyurans (Table 2).

2.4. DNA extraction, amplification and sequencing

Genomic DNA from fresh muscle tissue or testes was extracted and purified following PureGene (Gentra Systems) extraction protocols for animal tissue. Extracted DNA was quantified on a TD-360 Fluorometer (Turner Designs) and visualized on a 0.7% agarose gel stained with ethidium bromide to verify DNA quality. PCR reactions were conducted in a Stratagene RoboCycler thermal cycler with the following amplification profile: 10 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 30 s at the optimal annealing temperature, and 30 s at 72 °C and followed by an additional 7 min at 72 °C. Table 2 lists optimal annealing temperatures for each pair of primers.

Each 25 μl PCR reaction contained 200 μM of each dNTP; 300 nM each PCR primer; 1× PCR buffer (10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, Perkin Elmer); 1.25 U AmpliTaq Gold polymerase (Perkin Elmer); and template DNA (10–60 ng). PCR products were visualized on a 1.5% agarose gel to verify a single product of the correct size was amplified. Two microliters of ExoSAP-it (USB) were added to the remaining PCR product (~20 μl) and incubated at 37 °C for 15 min to remove residual primers and dNTPs, followed by 15 min at 85 °C to heat deactivate the enzymes. Amplicons were sequenced with Big Dye™

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